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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
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Monoclonal antibodies with specificity for fetal erythroid cells

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Monoclonal Antibodies with Specificity for Fetal Erythroid Cells

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Monoclonal Antibodies with Specificity for Fetal Erythroid Cells

1 Introduction

Social developments led to an increase of prenatal investigations. Amniocentesis or less frequently sampling of chorionic villi is performed in every tenth pregnancy for the prenatal analysis of, e.g. trisomy 21. The risk for a chromosomal defect increases with the age of the mother. This is why amniocentesis is performed in more than 50% of pregnant women aged 35 years or older. However, most children with chromosomal or genetic defects are still born by women under the age of 35, if the total number is taken into account. The probability for a trisomy 21 is 0.3% in fetuses of women aged 35 years and older. This has to be seen in the con-

text of a 0.5 % risk to induce an abortion by the amniocentesis procedure. From these numbers it is obvious that there is a great need for an alternative diagnostic procedure which yields the same results without bearing a risk for the unborn. One approach could be the isolation of fetal cells from maternal blood. This would eliminate risks for the fetus.

It was estimated that one fetal cell can be found in 10^5 to 10^7 maternal nucleated blood cells. Further investigations have shown, that in the presence of chromosomal aberrations more fetal cells can be detected in the maternal circulation. This raises the chance to detect an aneuploid fetus by non-invasive procedures.

Three different types of fetal cells have been identified in maternal blood: lymphocytes, trophoblasts and nucleated red blood cells (NRBCs).

Fetal lymphocytes have been detected still one to 5 years after childbirth. This longevity may interfere with the accurate diagnosis in following pregnancies.

Trophoblasts are attractive targets because they can be easily identified by their morphology. However, they can not be used for diagnostic purposes, because as placental cells they might differ from cells of the fetus: in about 1% of diagnosed chromosomal aberrations in trophoblasts the fetus turned out to be healthy.

Fetal nucleated red blood cells (NRBCs) appear early in the maternal circulation, however do not persist after birth. Since they have a nucleus they are preferred candidates for chromosomal analysis. However, they can not be distinguished easily and unambiguously from other blood cells. They are

identified through a marker profile, which is characteristic for erythroid precursor cells and which is different from other blood cell sub-populations. Blood cells are extensively characterized by so-called clusters of differentiation (CD) markers as defined at the 7th Workshop and Conference on Human Leukocyte Differentiation Antigens (Harrogate 2000). Immature erythroid cells express CD71 and they lack CD45 which is expressed on leukocytes. This knowledge can be used to distinguish erythroid precursor cells from other mononuclear cells.

In order to isolate and identify fetal cells (1 amongst 10^5 to 10^7 maternal nucleated cells) most stringent criteria have to be met. There is no cell surface marker available yet which is exclusively expressed on fetal NRBCs. For the enrichment of fetal cells usually immunomagnetic or flow cytometric cell separation techniques are used either alone or in combination. The results of the chromosomal or genetic analysis of the isolated cells has been compared with the results obtained with amniotic cells. Many investigations have shown the technical feasibility of the non-invasive approach with large cohorts.
However, the existing procedures are still not suitable for routine diagnosis. It has to be assured that the cells under investigation are unambiguously fetal cells. The definite identification of fetal cells can only be achieved by the recognition of a marker, which is exclusively found on fetal cells.

The lack of markers, which specifically identify fetal cells is the crucial obstacle for the development of a reliable non-invasive prenatal diagnos-

tic. The aim of this approach was to generate monoclonal antibodies that label exclusively fetal erythroid cells and thus allow a differentiation of fetal and adult blood cells.

2 Objective of the Invention

The objective of this invention is the generation of an antibody, which allows the discrimination between fetal and adult erythroid cells and the unambiguous identification of fetal cells. Fetal cells recognized by this antibody should possess an intact cell nucleus, express the CD71 antigen and miss the CD45 antigen in line with previously published results.

Cord blood cells carrying this antigen have been isolated. These mononuclear cells were further characterized for the expression of the CD71 antigen, the absence of the CD45 antigen and accordingly isolated. The immunization with these cells opens the possibility that besides antibodies against the "i" antigen also antibodies with specificities against new markers could be generated, which could be used to identify erythroid precursor cells.

3 Subject of Invention

The subject of the invention is the generation of monoclonal antibodies, which react specifically with fetal NRBCs, which express the CD71 antigen but lack the CD45 antigen.
For this purpose 5 mice have been immunized with isolated erythroid cells from cord blood (CD71+, CD45-), which carried the "i" antigen as defined by

the autoantibody described in DE 100 35 433. The spleen cells of the immunized mice were fused with a myeloma cell line to produce hybridomas according to standard procedures (*Schetters H*, Production of Monoclonal Antibodies, in: Methods of Immunological Analysis, *Masseyeff RF, Albert WH and Staines NA* (Eds.) Vol. 2, Ch. 4.3, 230-245, VCH Weinheim, 1993).

Short outline of invention

DESIGN AND METHODS:

Mice were immunized with flow sorted human cord blood cells (CD71+, antigen-i+, CD19- and CD45-). Hybridoma supernatants were screened on pooled mononuclear cord blood cells, whereas the corresponding amount of erythroid precursors was determined by cytochemical staining of blood smears. For the hybridoma screening a six-parameter flow cytometric analysis (four colours, forward and side scatter) was set up for the simultaneous identification of erythroid precursor cells, leukocytes, enucleated erythrocytes and for antibodies reacting specifically with fetal cells. Furthermore, immunohistochemical analyses have been performed with fetal blood smears and fetal liver sections from the 6^{th} up to 38^{th} week of gestation as well as with adult blood, normal adult bone marrow and adult erythrocytes as controls.

RESULTS:

A clone with specificity for a surface antigen exclusively expressed on fetal erythroid cells has been identified. The new antibody showed unaltered

binding to erythroid cells from fetal blood of early times of gestation (6th week) up to childbirth. Moreover, detailed examinations showed no surface reactivity with adult erythrocytes, erythroblasts or lymphatic and myeloid cells. This antibody did not react with cells of fetal haemolymphatic organs.

CONCLUSIONS:

The investigation showed that the new monoclonal antibody binds specifically fetal erythroid cells and thus can differentiate between fetal and adult red blood cells. Because of the expression of this fetal antigen in early stages of gestation a non-invasive prenatal diagnostic may be feasible. This antibody can be applied for different enrichment techniques and/or for the identification of fetal erythroid cells.

Example 1

Screening for Hybridomas Producing Antibodies Reacting Specifically with Fetal NRBCs

Since several thousand antibody producing hybridomas have to be screened to find a suitable clone a procedure has been set up permitting a high through-put whilst maintaining the required specificity. A six-parameter analysis (4 fluorescence channels, forward and side scatter) has been established, which enabled the simultaneous identification of erythroid precursor cells, the differentiation of leukocytes from enucleated erythrocytes and the identification of new antibodies in a single step. The analysed cells have been stained with a

nucleic acid dye (LDS751, Molecular Probes, cat# 7595) and have been incubated with antibodies of the cloned hybridomas. These antibodies were subjected to a reaction with an antibody directed against them, which was labelled with a fluorescent dye (FITC) (Goat anti mouse IgG (H+L)-FITC, Caltag Laboratories, cat# M35001). In later experiments for antibody characterization the antibodies have been labelled directly with FITC.

The identification of the erythroid precursor cells is possible due to their light scatter characteristics and by their binding of phycoerythrin labelled CD71 specific antibodies (CD71 PE, Diatec, cat# 3212). Leukocytes could be discerned by their binding to allophycocyanin labelled CD45 specific antibodies (CD45 APC, BD Pharmingen, cat# 555485). Nucleated and enucleated erythroid cells can be distinguished by their binding or absence of binding of the nucleic acid dye. With this procedure it is possible to identify antibodies binding to the intended target cells, i.e. fetal NRBCs, without cross-reaction towards adult erythrocytes or leukocytes (Fig. 1).

Example 2

Exclusion of Antibodies Reacting with Antigens on Adult Erythrocytes Including Common Blood Group Antigens

Blood group antigens can be found on adult erythrocytes and their precursors in large amounts. Therefore, they might induce a major immune response when used as antigens. Antibodies against these blood group antigens are not suitable for the iden-

tification of fetal cells. In order to exclude antibodies binding to antigens on adult erythrocytes including blood group antigens, their binding specificity towards fetal cells is investigated after absorption on erythrocytes. Erythrocyte with the blood group AB Rh+ have been harvested and stabilized with a reagent supplied by Meridian Diagnostics Europe, Bad Homburg. The antibodies under investigation have been incubated with increasing numbers of erythrocytes and tested before and afterwards for their binding activity for target cells. Reactivity of antibodies towards blood group antigens was thought to be absent, when the intensity of the binding to CD71+, CD45- nucleated erythroid precursor cells was unchanged after the incubation with the erythrocytes (Fig.2). Antibodies selected that way must not react with adult blood cells to enable the correct identification of fetal erythroid precursor cells (Fig. 3).

Example 3

Specificity Testing of a Selected Monoclonal Antibody

A hybridoma clone producing a monoclonal antibody of the IgM isotype showing the required binding characteristics in the screening procedure could be identified. It has the designation 4B9 and is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig). A second antibody 4B8 recognizing the same epitope is mentioned in figures 2 and 3.

Fetal and adult erythroblasts strongly and specifically express glycophorin-A and, therefore, can be

identified through this marker protein. The binding of the monoclonal antibody to these cells was visualized by a immunofluorescence double stain.

Protocol for Immunofluorescence Stain

1. Fix cytospins or frozen tissue sections in acetone for 10 min
2. Dry for 5 min
3. Apply monoclonal antibody against glycophorin-A ,DAKO M0819 diluted 1:100 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 60 min
4. Rinse with PBS
5. Apply goat anti-mouse antibody F(ab) fragment, Alexa Fluor 488 (Molecular Probes A-21044), green, diluted 1:100 in PBS for 60 min
6. Rinse with PBS
7. Apply monoclonal antibody 4B9 (hybridoma supernatant) for 60 min
8. Rinse with PBS
9. Apply goat anti-mouse IgM, Alexa Fluor 594 (Molecular Probes A-21044), red, for 60 min
10. Rinse with PBS
11. Stain cell nuclei with DAPI (Molecular Probes), blue, diluted 1:300 in PBS for 3 min
12. Rinse with PBS
13. Cover with fluorescence medium (S3023, DAKO)
14. Visualize with "Universalmikroskop Axioplan", Carl Zeiss, using filter sets 02, 10 and 15 and photograph with a digital camera system, e.g. Visitron Systems GmbH

PBS: 8 g NaCl, 1.3 g Na_2HPO_4, 4 g NaH_2PO_4 in 1 l H_2O, pH 7.4

An immunoenzymatic method has also been used:

Protocol for Alkaline Phosphatase Anti-alkaline Phosphatase (APAAP) Stain

1. Fix cytospins or frozen tissue slices in acetone for 10 min
2. Dry for 5 min
3. Incubate with monoclonal antibody 4B9 (hybridoma supernatant) for 30 min
4. Rinse with Tris buffered saline (TBS)
5. Incubate with APAAP complex (D0651, DAKO), diluted 1:25 in TBS/HS (inactivated human serum) for 30 min
6. Rinse with TBS
7. Repeat steps 5-7 twice for 10 min each
8. Rinse with TBS
9. Develop slides with substrate
   i. Prepare solution A: Mix 18 ml 0.2 mol/l 2-amino-2methyl-1,3-propandiol with 50 ml 0.05 mol/l Tris buffer, pH 9.7 and 600 mg NaCl. Add 28 mg levamisol.
   ii. Prepare solution B: Dissolve 35 mg naphthol AS-bi-phosphate in 0.42 ml N,N-dimethylformamide.
   iii. Prepare solution C: Mix 0.14 ml 5% New Fuchsin with 0.35 freshly prepared 4% sodium nitrite. Stir for 60 sec.
   iv. Mix solution A with solution B, then add solution C. Adjust the pH to 8.7. Mix, filter and apply to slides.

v. Incubate for 10-20 min at room temperature.
vi. Rinse with tap water.

vii. Counter stain with Meyer's acid Haemalaun for 5 min.
viii. "Blue" in tap water for 10 min and cover with Kaiser's glycerol gelatine.

TBS (Tris buffered saline): Dissolve 43.33 g NaCl and 39.40 g Tris-HCl in 5 l H_2O dest. Adjust pH to 7.4 with NaOH.
TBS/HS: 9 parts TBS + 1 part inactivated human serum

Negative controls: monoclonal antibody of identical isotype or murine hyper-immune serum.

Results

- The 4B9-reactive antigen was strongly expressed on the surface of fetal erythroblasts. This could be demonstrated in a double stain of fetal cells from the 6. up to the 38. week of gestation: the antibody 4B9 recognised all glycophorin-A positive fetal erythroblasts (Fig. 4).
- Erythroblasts in normal adult bone marrow were negative for 4B9. In contrast, all erythropoietic cells were positive for glycophorin-A (Fig. 5). Only in 1 of 32 cases a intracellular granular expression in the cytoplasm of early basophile erythroblasts was seen.
- The 4B9 reactive antigen was not found on adult and fetal liver hepatocytes. Kupffer

cells, macrophages, endothelial and sinusoidal cells were also negative (Fig. 6).

- A detailed analysis of haemolymphatic cells in adults showed the absence of reactivity in lymphatic and myeloic cells.
- All haemolymphatic organs of the fetus were negative. This applies for lymphatic as well as myeloic cells.

Table 1. Detailed results of the reactivity of the monoclonal antibody 4B9

Cell or tissue	Adult (n positive / n samples)	Fetal (n positive / n samples)
Granulopoiesis		
Neutrophils		
Segmented	0/8	0/14
Rodforms	0/8	0/14
Metamyelocytes	0/8	0/14
Myelocytes	0/8	0/14
Promyelocytes	0/8	0/14
Eosinophils		
Rodforms	0/8	0/14
Metamyelocytes	0/8	0/14
Myelocytes	0/8	0/14
Promyelocytes	0/8	0/14
Basophils	0/8	0/14
Monocytes		
Mature monocytes	0/8	0/14
Promonocytes	0/8	0/14
Myelocyte	0/8	0/14
Macrophages	0/8	0/14
Thrombocytopoieses		
Platelets	0/8	0/14
Megakaryocytes	0/8	0/14
Megakaryoblasts	0/8	0/14

Erythrocytopoiesis		
Erythrocytes	0/8	14/14
Reticulocytes	0/8	10/10
Normoblasts	0/8	4/4
Euchrom. Erythroblasts	1/32	5/5
Polychrom. Erythroblasts	0/8	10/10
Basophilic erythroblasts	0/8	4/4
Proerythroblasts	0/8	4/4
Lymphocytopoiesis		
B lymphocytes	0/8	0/14
Plasma cells	0/8	0/14
T lymphocytes	0/8	0/14
Hepatocytes	0/8	0/4
Kupffer cells	0/8	0/4
Other hepatic cells	0/8	0/8

Figures

Figure 1
Mononuclear cord blood cells were stained with labelled antibodies (anti CD45, anti CD71 and the antibody under investigation, 4B9) and a DNA dye. Antibody binding was measured with a flow cytometer.

a) Erythroid precursor cells characterized by means of their light scatter properties (Region 1),
b) Differentiation between enucleated erythrocytes and nucleated cells (Region 2), and
c) Differentiation between CD71 positive nucleated erythroid cells (Region 3) and CD45 positive leukocytes.
d) Binding of antibody 4B9 to CD71 positive but CD45 negative nucleated cells (Region 4).

Figure 2
Absorption of monoclonal antibodies 4B8 and 4B9 with adult erythrocytes, followed by the determination of their binding capability on cord blood cells. For positive and negative controls antibodies against CD71 and glycophorin A were used.

Figure 3
Flow cytrometric investigation of the binding of the monoclonal antibodies 4B8 and 4B9 on cord blood cells and adult blood cells.

a) This histogram shows unstained, negative cord blood cells (grey) and cord blood cells stained with labelled antibodies 4B8 (red) and 4B9 (yellow).
b) Antibodies 4B8 and 4B9 do not bind to adult blood cells.

Figure 4
Immunofluorescent and immunoenzymatic analyses of fetal blood cells.

a) - e) Glycophorin A-positive (green fluorescence) fetal erythropoietic cells express the 4B9 antigen (red fluorescence). Cell nuclei are stained with DAPI (blue). Nucleated and enucleated red blood cells are positive for the 4B9 antigen.

f) - h) Positive immunoenzymatic APAAP-staining of the monoclonal antibody 4B9 on fetal red blood cells. Magnifications: f): × 100; g), h): × 75

Figure 5
APAAP-staining of adult bone marrow cells.

a), b) Chromatin-dense erythropoietic cells (arrow) are negative after a APAAP-reaction with antibody 4B9. Magnification: a) × 200, b) × 400

Figure 6
Immunofluorescent analysis of frozen liver sections.

a) No reaction with labelled antibody 4B9 on adult liver cells. Cell nuclei are stained with DAPI (blue). Magnification: × 100

b) Glycophorin-A antibody (green fluorescence) shows a positive reaction on adult erythrocytes. Cell nuclei are stained with DAPI (blue). Magnification: × 200

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Claims

1. Monoclonal antibody reacting with a surface antigen present on fetal nucleated red blood cells including their precursor cells, but not with surface antigens on adult erythroid cells, furthermore not reacting with surface antigens on other fetal and adult cells such as neutrophils, eosinophils, basophils, monocytes and their precursor cells, nor with platelets, megakaryocytes and megakaryoblasts, not with lymphocytes and plasma cells, nor with Kupffer cells, hepatocytes, endothelial cells and sinusoidal cells.

2. Monoclonal antibody reacting with all fetal nucleated erythroid cells but not with adult erythroid cells and erythrocytes expressing glycophorin-A.

3. Monoclonal antibody which reacts with fetal nucleated erythroid cells expressing the CD71 antigen but being negative for the CD45 antigen.

4. Antibodies according to at least one of claims 1 to 3.

5. Surface antigen on fetal nucleated red blood cells recognized by a monoclonal antibody as characterized in one of claims 1 to 4.

6. Use of a monoclonal antibody according to one of claims 1 to 4 for the detection and identification of fetal nucleated red blood cells in a sample, preferably in maternal blood.

7. Use of a monoclonal antibody according to one of claims 1 to 4 for the detection and identification of fetal nucleated red blood cells in a sample, preferably in maternal blood, for the purpose of analysis of chromosomal aberrations and genetic defects of the fetus.

8. Use of an antibody according to one of claims 1 to 4 for the enrichment and / or isolation of cells carrying the antigen.

9. Use of an antibody as characterized in claims 1 to 4 for the elimination of cells carrying the antigen from a sample.

10. Use according to one of claims 6 to 9, characterized in that cells binding the monoclonal antibody are separated by flow cytometry, solid phase separation, immunomagnetic bead separation, panning on plastic surfaces, or the like.

11. Method for detection, identification, enrichment, isolation and/or elimination of fetal nucleated red blood cells in or from a sample, preferably maternal blood.

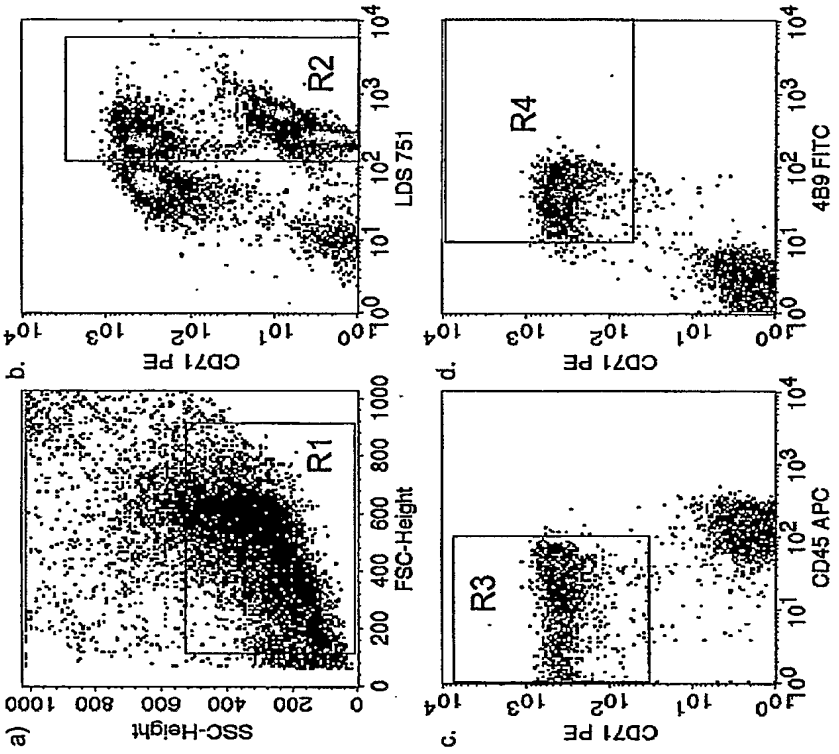


FIGURE 1

FIGURE 2

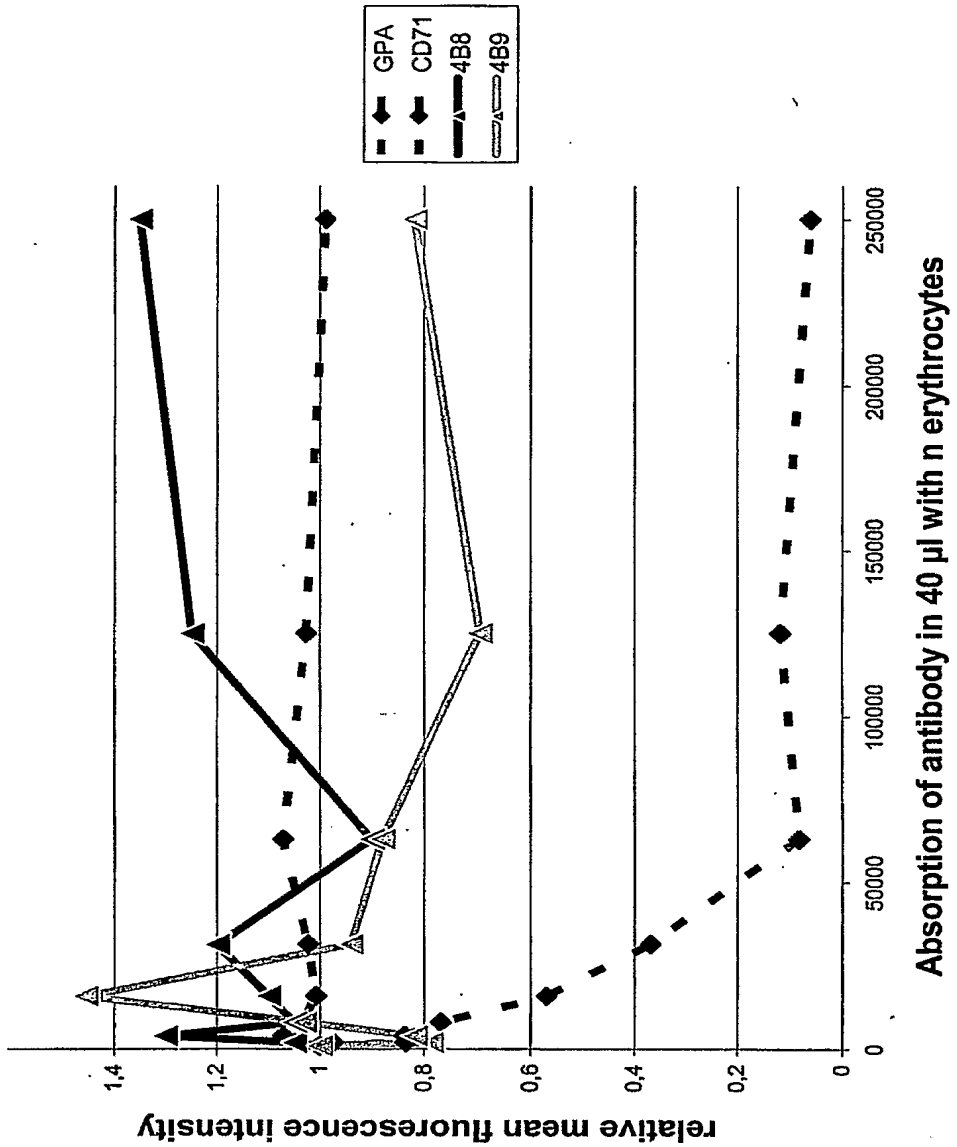
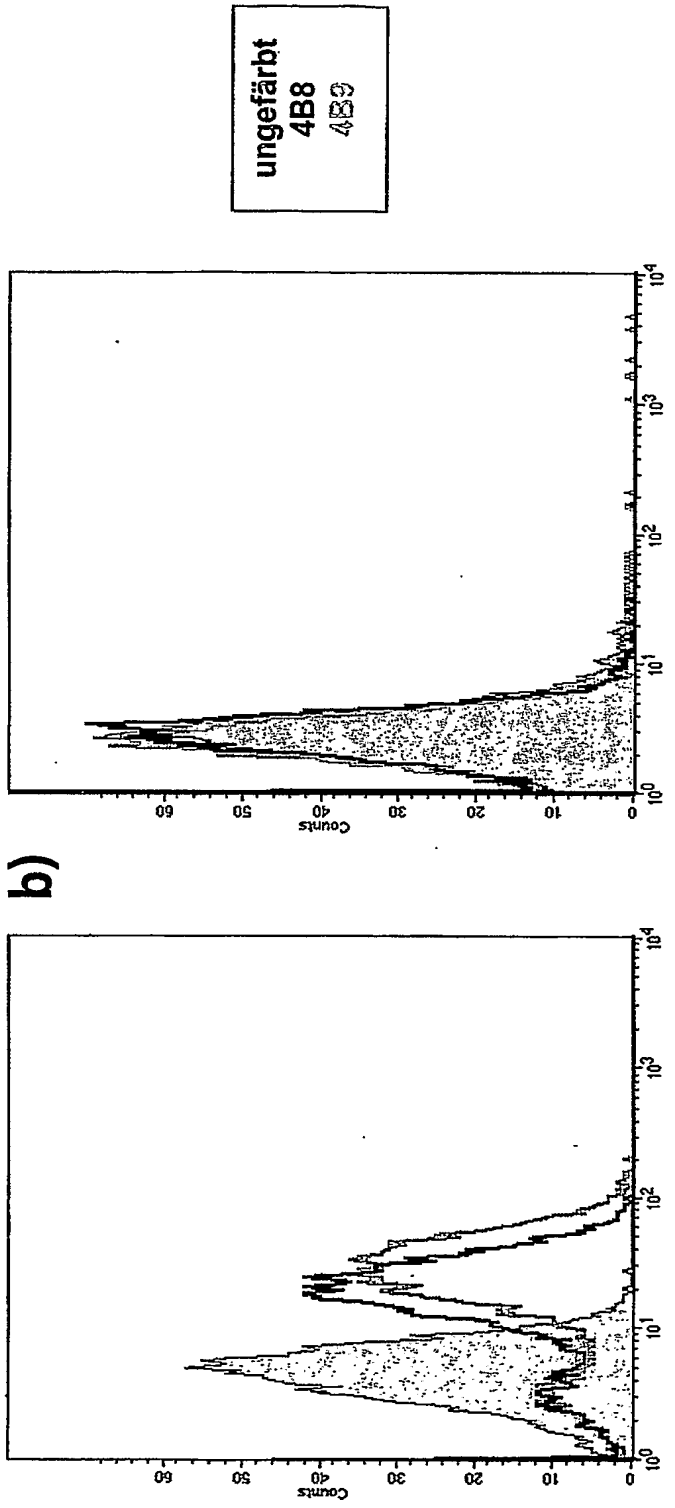

GPA
CD71
4B8
4B9
relative mean fluorescence intensity
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
50000
100000
150000
200000
250000
Absorption of antibody in 40 µl with n erythrocytes

FIGURE 3


ungefärbt
4B8
4B9
a)
b)
Counts
0
10
20
30
40
50
60
10^0
10^1
10^2
10^3
10^4

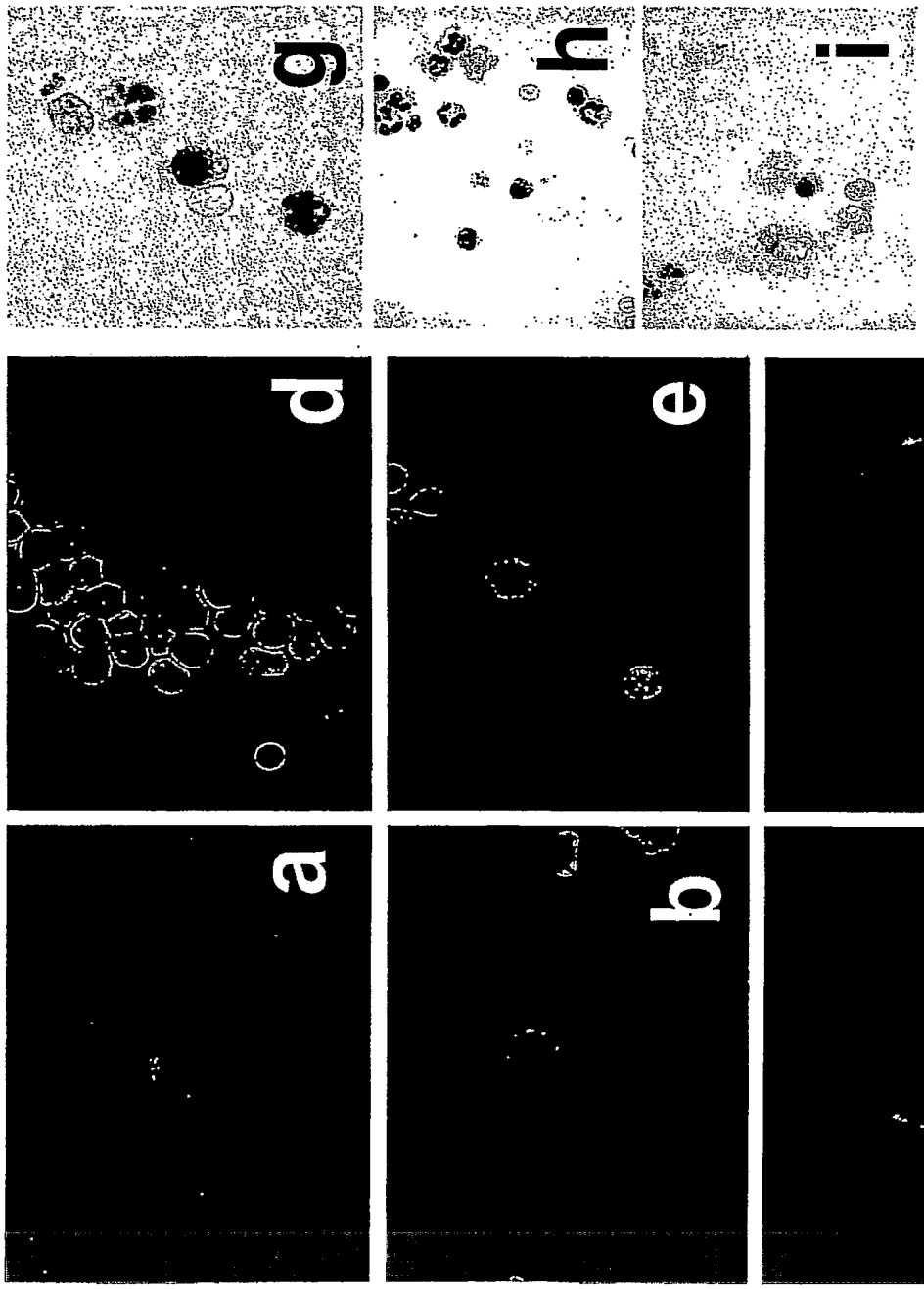


FIGURE 4

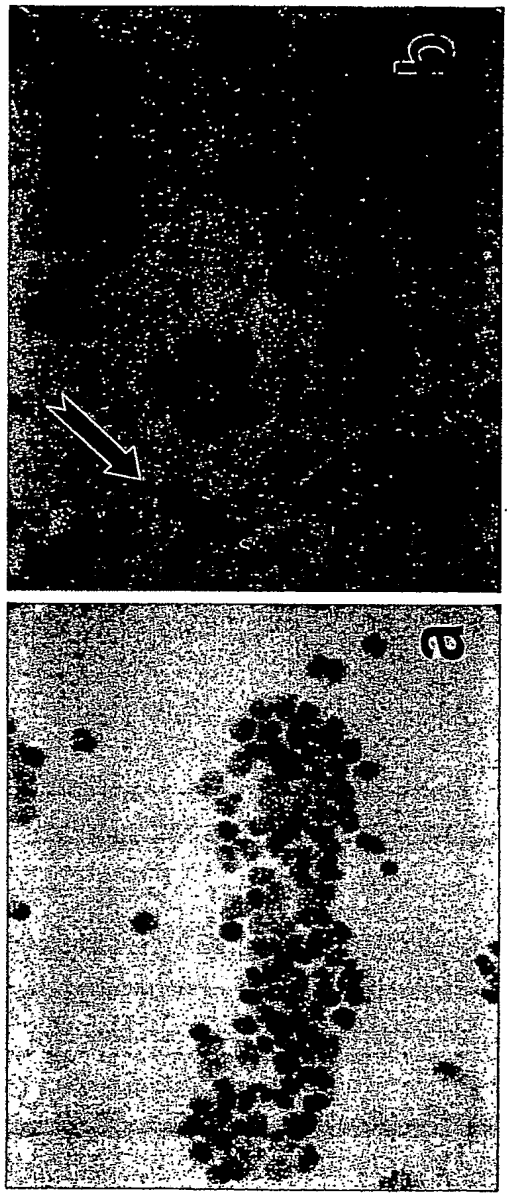


FIGURE 5

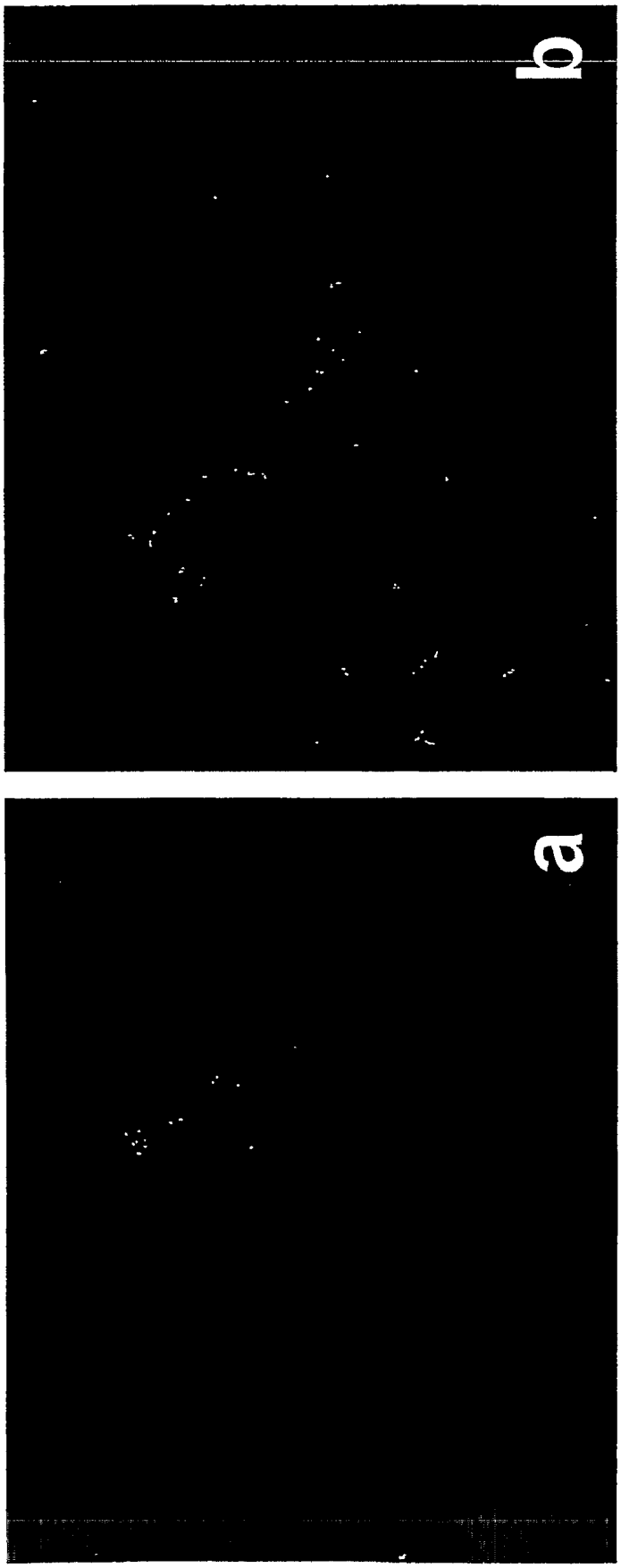


FIGURE 6